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Radiolabeled Exosomes for the Early Detection of Metastases and to Predict Breast Cancer Premetastatic Niche

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14. ABSTRACT Since current diagnostic tools fail to detect breast cancer (BC) metastatic spread at a very early stage, we proposed a a new approach for the early detection of metastatic disease in BC patients using cancer cell derived particles known as exosomes as a guide. We hypothesized that exosomes tagged with appropriate radionuclides could be used as molecular imaging probes to study and non-invasively image metastatic spread, and to detect pre-metastatic niches using single-photon emission computed tomography (SPECT) or Positron emission tomography (PET). **D**uring this report period we have: 1) isolated and characterized exosomes from a series of BC cell lines of different metastatic capacity and tropism, 2) radiolabeled isolated BC exosomes with different radionuclides, such as ^{111}In, ^{64}Cu, ^{89}Zr. We plan to 3) study the uptake and subsequent intracellular transport of radiolabeled exosomes by different cell types involved in pre-metastatic niche formation, 4) to study the in vivo stability, pharmacokinetics and biodistribution of radiolabeled exosomes following intravenous administration in naive and pathological mouse models. 5) Study the biodistribution and tumor uptake of radiolabeled exosomes (derived from peripheral blood of

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DOD Grant/Contract (Award Number W81XWH-13-1-0248)

YEAR 1 RESEARCH REPORT

Grant Title: Radiolabeled exosomes for the early detection of metastases and to predict breast cancer pre-metastatic niches

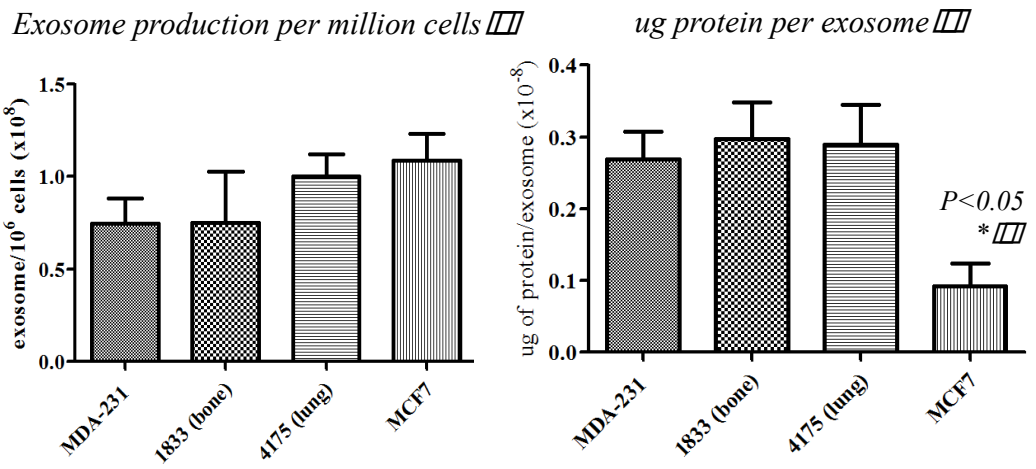
INTRODUCTION:

This grant is focused on the early detection of metastatic disease in patients with breast cancer (BC). This project takes advantage of the breakthrough knowledge in tumor-derived exosome tropism and exploits recent advances in the development of radioisotope based molecular imaging probes. We believe that non-imaging techniques, either single-photon emission computed tomography (SPECT) or positron emission tomography (PET) may offer an advantage to detect exosome distribution in metastatic organs and thus detect pre-metastatic niches *in vivo*. Our goal is to provide for the first time a framework for the prediction of metastatic spread and the diagnosis of pre-metastatic niche formation in BC based on preclinical studies using radiolabeled exosomes.

Summary of the tasks/aims proposed and achievements:

Specific Aim 1. To isolate and characterize fluorescently labeled exosomes from a series of BC cell lines of different metastatic capacity and tropism (timeframe, e.g., months 2-18). Dr. Lyden's group was and continues to be responsible for this task.
Specific Aim 1.1 To purify exosomes derived from MCF-7 and MDA-231 (available in 3 different clones and characterize, size, number and protein concentration of exosomes (timeframe, months 2-10).
Specific Aim 1.2 To prepare fluorescently labeled exosomes and determine the biodistribution in mice. 4 different exosome preparations will be studied in 40 mice (timeframe, months 2-10).

Figure 1. Analysis of exosome secretion in breast cancer models. The graph on the left represents exosome production per million BC cells. Cells were cultured for four days with exosome-free media followed by exosome isolation. Exosome numbers were quantified using Nanosight analysis. The graph on the right represents the amount of exosome protein, measured in micrograms by BCA assay per exosome. Exosome numbers were quantified using Nanosight analysis.



We have successfully isolated exosomes from both models (MCF-7 and MDA-231) and characterized their properties by Nanosight and mass spectrometry. Subtask 1.1 has been performed completely and we are waiting for ACURO approval to perform subtask 1.2. Our data demonstrates that highly malignant models (MDA231 and the BC cell line variants with tropism to the bone (1833) and to the lung (4175) secrete similar amounts of exosomes compared to the non-metastatic cell line MCF7. Importantly, we have observed that the amount of protein per exosome in highly malignant MDA231 models is significantly increased suggesting that exosome cargo could be involved in BC cell malignancy (Fig. 1). We have used 3 different models tropic to the lung, bone or brain. Analysis of BC exosome cargo by mass spectrometry demonstrated that there is a proteomic signature associated with the metastatic organotropism of these cell lines (data not shown, data is in the bioibformatics pipeline, being analyzed). We have developed and improved the protocol for labeling of exosomes with fluorescent dyes. Our data show that exosomes can be fluorescently labeled using red and green PKH membranes dyes as well as using near-infrared fluorescent dyes (i.e. CellVue). Our data demonstrate that we have an effective way to track exosomes that will allow us to compare with radioactively-labeled exosomes to perform the *in vivo* analyses.

Specific Aim 2: To develop procedures and protocols to radiolabel exosomes derived from BC cell lines, MCF-7 and MDA-231with different radionuclides in order to achieve radiolabeled exosomes of high specific activity, stability and specificity (timeframe, months 4-24).

Specific Aim 2.1. To radiolabel isolated BC exosomes with different radionuclides, such as ^{111}In, ^{64}Cu, ^{89}Zr
Specific Aim 2.2. To optimize the radiolabeling protocol in order to achieve a radioactive probe of high specific activity, stability and specificity.

Dr. Vallabhajosula's group is responsible for this task. He has made progress analyzing the best method to label exosomes radioactively and compare with our

results using fluorescently labeled exosomes. We are in a good position to perform further analyses and complete the proposed experiments in year 2.

Results:

Specific Aim 2.1. ^{111}In-oxine (non-specific blood cell radiolabeling agent).

Experimental part:

Exosomes isolated from MDA-MB-231 were used. Different exosome preparations have been tested, each containing different amount of particles: a) 20×10^7, b) 200×10^7, c) 3×10^9, d) 6×10^9 and e) 60×10^9 particles in a PBS solution (100uL). Labeling was performed following a protocol established in Nuclear Medicine practice for labeling of autologous white blood cells and platelets.
Briefly, 100uL of exosome solution was mixed with ^{111}In-oxine (0.1-0.5 mCi). Mixture was incubated at RT for 30 min while gently swirled periodically on a shaker. Following incubation, unbound ^{111}In-oxine was removed by centrifugation at 750g for 2 min with Sephadex containing spin columns pre-conditioned with PBS. Labeling efficiency was determined by measuring the amount of radioactivity for the exosome pellet and the unbound radiotracer. Radiochemical purity (RCP) was determined by instant-thin layer chromatography (ITLC) on silica gel strips (and high-pressure liquid chromatography (HPLC) using a size-exclusion column.
ITLC: SG strips (Biodex) and DTPA 5mM as mobile phase.
HPLC: Polysep GFC-P 4000 column (Phenomenex) and 0.1 N phosphate buffer pH 7.5 as mobile phase; isocratic elution for 40 min; Flow rate: 0.5 mL/min; UV: 220 nm; t_R for unlabeled Exosomes: 13 (±0.5) min

Results:
HPLC analysis of radiolabeling mixtures for d) and e) exosome preparations showed (radio)peaks with t_R= 13 (±0.5) min that can be attributed to the formation of ^{111}In-labeled exosomes. However, the purification method used for radiolabeled exosomes was not efficient in separating unbound ^{111}In-oxine from exosomes and thus initial HPLC findings were not confirmed.

Future plans:
i) To test radiolabeling with higher amounts of ^{111}In-oxine and higher concentrations of exosomes.
ii) To test different and more efficient purification methods such as ultra-centrifugation at 100,000g for 2-3 hours and/or gel filtration on PD-10 columns.

Specific Aim 2.2. Iodine-131 (radiolabeling of Tyrosine and Histidine residues)

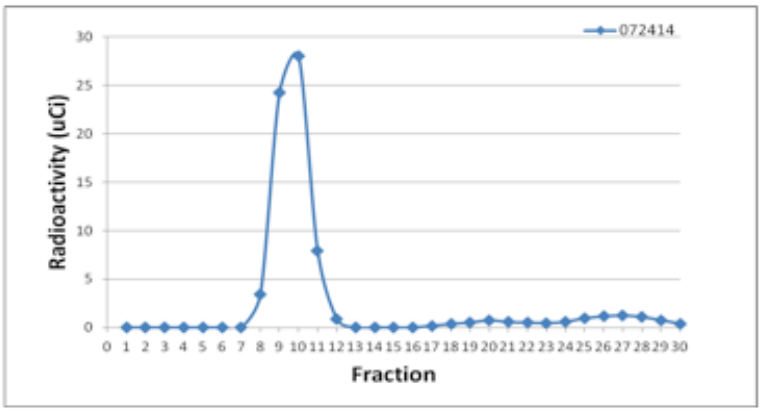
Experimental part:

Exosomes isolated from MDA-MB-231 were used. Different exosome preparations have been tested, each containing different amount of particles: a) 1.2×10^9, b) 6×10^9, c) 20×10^9 and d) 50×10^9 particles in a PBS solution (100uL). Labeling with I-131 was performed

using the Iodogen method and PIERCE® pre-coated iodination tubes. $Na^{131}I$ of high radiochemical purity was supplied by Perkin Elmer.
In a final volume of 0.15-0.35mL, exosomes were mixed with $Na^{131}I$ (0.3-1 mCi) in 25mM Tris HCl pH 7.5, 0.4M NaCl buffer. Mixtures were incubated at 33°C for 1.5 h while gently swirled periodically on a shaker. Reaction was quenched by addition of an excess (50uL; 10mg/mL) of p-hydroxyphenylpropionic acid that serves as free I-131 scavenger. Unbound I-131 was removed by size exclusion chromatography on PD-10 columns pre-conditioned with 25mM Tris HCl pH 7.5, 5mM EDTA, 0.4M NaCl, 0.25% BSA buffer that serves also as the elution buffer (0.5mL fractions collected). Centrifugation at 750g for 2 min with Sephadex containing spin columns was also tested but without success to remove unbound I-131. Labeling efficiency and Radiochemical Purity (RCP) were determined by instant-thin layer chromatography (ITLC) on silica gel strips (and high-pressure liquid chromatography (HPLC) using a size-exclusion column.
ITLC: SG strips (Biodex and Agilent Technologies) and 85% MeOH or PBS as mobile phase.
HPLC: Polysep GFC-P 4000 column (Phenomenex) and 0.1 N phosphate buffer pH 7.5 as mobile phase; isocratic elution for 40 min; Flow rate: 0.5 mL/min; UV: 220 nm; t_R for unlabeled Exosomes: 13 (±0.5) min

Results:
Separation of radioiodinated exosomes from unbound I-131 with PD-10 columns was evident in all cases. See representative PD-10 elution profile below (radioiodinated exosomes in fractions F8-F11):

Figure 2. Analysis radioiodinated exosomes from breast cancer models. The graph depicts successful separation of radioiodinated exosomes from unbound I-131 with PD-10 columns.



Labeling efficiency was found to be proportionally dependent on the concentration of exosomes used in the reaction mixture. HPLC and TLC analysis showed higher labeling yields (80-90%) for the exosomal preparations containing 20-50×10^9 particles. RCP was

>95% for the high exosome content preparations and 70-80% for the rest. The specific activity of final purified products ranged in 3-10 uCi/10^9 particles or 2-8 uCi/ug of protein.

Specificity: Initial uptake studies in cells (mast cells) following incubation for 1-2 h didn't confirm the specificity of the radioiodinated exosome preparations. However, alternative cell uptake protocols need to be tested.

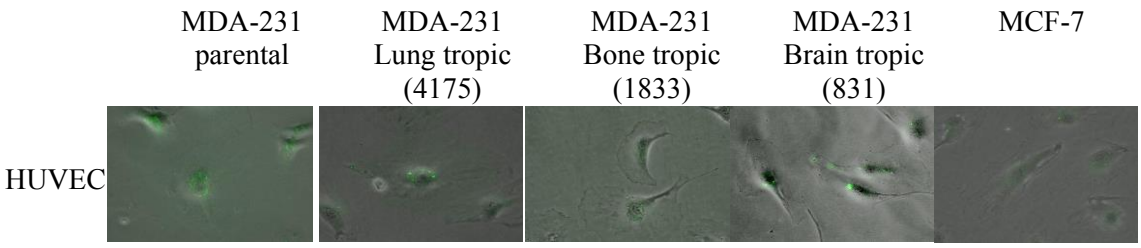
Future plans:

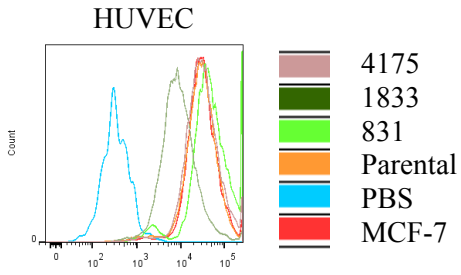
i. To test the specificity of radioiodinated exosomes using different types of cells and longer incubation times based on the experience with the fluorescently labeled exosomes.
ii. To study the biodistribution of radioiodinated exosomes in animals.
iii. To develop new protocols to radiolabel exosomes with radiometals such as Zr-89, In-111 or Cu-64 following modification with appropriate metal chelators (e.g. DFO, DOTA).

Specific Aim 3. To study the *in vitro* uptake and subsequent intracellular transport of radiolabeled exosomes by different cell types, known to participate in the pre-metastatic niche formation mechanism, such as the primary tumor cells, BM cells, fibroblasts (NIH/3T3, lung) and endothelial cells (breast, lung, HUVEC). The binding, internalization and intracellular trapping of radiolabeled exosomes and fluorescence labeled exosomes will be compared (timeframe, months 8-24).

Specific Aim 3.1 Determine the kinetics of labeled exosomal binding (and internalization) to tumor cells, BM cells, fibroblasts and endothelial cells.
Specific Aim 3.2 Evaluate the transference of exosomes from BC to stromal cells in vitro
Specific Aim 3.3 Determine the specific populations of bone marrow cells that can specifically take up exosomes from BC cell lines using flow cytometry.

Figure 3. Breast cancer exosomes are efficiently uptaken by stromal cells.





We have initiated the analysis of tumor exosome uptake *in vitro*. For this purpose exosomes were first labeled fluorescently and incubated with fibroblast or endothelial cells (Fig. 3). We have used different models of stromal cells to perform these assays and incubated these cells with exosomes derived from MDA231 BC. Our data demonstrate the ability of these cell types to uptake BC exosomes. Specifically, we have analyzed the ability of NIH3T3 fibroblast to uptake exosomes (data not shown) and we observed that over 90% of cells are able to uptake exosomes over 24 hours of incubation with 10ug/ml BC exosomes. Similarly, our analysis in HUVEC endothelial cells demonstrate that over 98% of cells uptake exosomes efficiently (Fig. 3). These data support the idea that BC exosomes can be transferred to stromal cells and tracked *in vitro*. As soon as we receive ACURO approval for animal studies, we will analyze the different subpopulations of cells in the BM that are uptaking tumor exosomes. We will perform these approaches *in vivo* during the second year of funding.

Specific Aim 4. To study the *in vivo* stability, pharmacokinetics, and biodistribution of radiolabeled exosomes following intravenous injection in naive and breast cancer high malignant (MDA-231) and low malignant (MCF-7) human breast cancer mouse models (timeframe, months 13-36).

Research staff from Dr. Vallabhajosula and Dr. Lyden will be working together on this task.

The design of in vivo studies in animal models depends very much on the success of in vitro stability studies and in vitro tumor cell binding studies with radiolabeled exosomes. A tentative plan is presented here to assess biodistribution.

Specific Aim 4.1 Identify 5 different radiolabeled exosomal preparations with optimal radiochemical purity, specificity and specific activity.

Specific Aim 4.2 Perform in vivo biodistribution studies at different time points (10 min,

1, 2, 4, 8, 24 hours) following administration of 0.1-0.3 mCi of radiolabeled (111In, 64Cu or 89Zr) exosomes based on μSPECT/PET/CT imaging system

Specific Aim 4.3 Based on imaging studies identify 2 time points for tissue distribution studies. Following sacrifice of mice (5 mice/timepoint/radiotracer), perform biodistribution by counting radioactivity in different tissue samples.

Specific Aim 4.4 Compare the biodistribution data obtained with radiolabeled exosomes with similar biodistribution data obtained using fluorescent labeled exosomes

All these experiments will be performed during the second year, all our data from Aims 1 to 3 supports the idea that these analyses will be starting and successful upon receiving ACURO approval.

Specific Aim 5. Following establishment of a successful radiolabeling protocol for exosomes derived of BC cell lines, a) develop protocols for the preparation of radiolabeled exosomes isolated from the circulation of BC patients, and b) study the biodistribution and tumor uptake of radiolabeled exosomes in BC xenografts.

Research staff from Dr. Vallabhajosula and Dr. Lyden will be working together on this task.

Specific Aim 5.1 Purify exosomes from human blood (BC patient) and characterize, size, number and protein concentration of exosomes

Specific Aim 5.2 Prepare radiolabeled exosomes (based on the best labeling method identified in specific aim 2)

Specific Aim 5.3 Perform in vitro cell binding studies with tumor cells, BM cells, fibroblasts and endothelial cells.

Specific Aim 5.4 Perform in vivo imaging studies and kinetics of distribution studies in BC high malignant (MDA-231) xenografts.

Specific Aim 5.5 Correlate the biodistribution data with tissue distribution data obtained with fluorescent labeled exosomes.

Due to high variability of yields and lack of reproducibility we have decided for now not to pursue the analysis of BC patient exosomes. Therefore, all the experiments performed as part of this grant will be performed with exosomes isolated from *in vitro* organotropic models of human breast cancer.

KEY RESEARCH ACCOMPLISHMENTS:

- We determined for the first time that the amount of protein per exosomes is increased in exosomes derived from triple negative breast cancer cell lines.
- We have established that exosomes can be efficiently labeled with fluorescent and near-infrared dyes.
- We have demonstrated that tumor-secreted exosomes can be transferred to fibroblast, endothelial and bone marrow-derived cells.

- Importantly, we have analyzed the proteomic content of BC models and developed a database of protein secreted in exosomes.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- Dr. Lyden has presented preliminary data from some of these studies in 10 national and international meetings this past year. The top five meetings were:
    - San Antonio Breast Cancer Conference, San Antonio, Texas, USA, Dear December 10-13, 2013.
    - AACR sponsored meeting on the tumor microenvironment in San Diego, California, USA, Feb 26 -March 1, 2014
    - International Society of Extracellular Vesicles, Rotterdam, The Netherlands, April 30-May 3, 2014
    - The TMEN NCI Meeting, June 10-12, 2014, Weill Cornell Medical College, New York, NY, USA (Dr. Lyden organized and hosted this meeting)
    - International Metastasis Meeting in Heidelberg, Germany, June 27 - July 2, 2014

- Dr. Peinado has presented as invited Speaker at the following meetings:
    - V European Melanoma Workshop, Marseille, France, July 2013.
    - Symposium on exosomes and microvesicles, UIMP, Spain, September 2013.
    - University of Vermont, USA, October 2013.
    - Extracellular Vesicles as Therapeutics. Rhode Island, USA, June 2014.
    - V GEM and 6th European Melanoma Workshop. Canary Island, Spain, July 2014.

CONCLUSION:

We have confirmed that BC exosomes can be efficiently labeled fluorescently and tracked *in vitro*. The biological data confirm that highly malignant breast cancer cell lines secrete exosomes with increased amount of protein and can be transferred to stromal cells. The development of a molecular imaging technique that could predict metastatic niches would allow foreseeing metastatic spread of tumors and metastatic organ. Our test with radiolabeled exosomes suggest that BC exosomes could be used as radioactive probes for non-invasive molecular imaging studies using single photon emission computed tomography (SPECT) and positron emission tomography (PET) in order to predict metastatic spread and metastatic niches. We propose to test this hypothesis during

next year in animal models of breast cancer metastasis using high resolution (0.7-1.3 mm) microSPECT/PET/CT trimodal imaging system and autologous exosomes labeled with SPECT and PET radiometals such as indium-111, copper-64 and zirconium-89.

All these data will impact cancer research to design radioactive probes based in synthetic exosomes with specific cargo that may predict pre-metastatic sites in the clinic. This could result in valuable nuclear imaging system to predict metastasis in BC patients. This could result in valuable combination therapy for the management of cancer patients. Ultimately, these studies will support the use of exosomes in the clinical setting by detecting pre-metastatic niches in breast cancer patients.

REFERENCES: Not applicable. No references are associated with this report.

APPENDICES: Not applicable. No appendices are attached to this report.

SUPPORTING DATA: Figures 1-3.